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吸入性麻醉劑七氟烷對人類多形核白血球之影響
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吸入性麻醉劑七氟烷對人類多形核白血球之影響

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Abstract

Sevoflurane is an inhalation anesthetic using for general anesthesia. Several studies have demonstrated that reactive oxygen species (ROS) exist in cardioprotection when preconditioned with sevoflurane. Moreover, sevoflurane can also directly trigger the formation of peroxynitrite. Up to now, information pertinent to the effect of sevoflurane on cellular injuries in human polymorphonuclear neutrophils (PMN) is scant. In this study, we demonstrated that sevoflurane significantly increases intracellular H_2O_2 and/or peroxide, superoxide, and nitric oxide (NO) in PMN within 1 h treatment. Intensification of intracellular glutathione (GSH) depletion in PMN has been demonstrated with the presence of sevoflurane. The inhibition of sevoflurane-mediated intracellular H_2O_2 and/or peroxide in PMN by catalase, mannitol, dexamethasone, N-acetylcysteine (NAC) and trolox, but not superoxide dismutase (SOD) pretreatment, was observed. Among them, catalase has the best effect scavenging intracellular H_2O_2 and/or peroxide, suggesting that H_2O_2 is the major ROS during sevoflurane treatment. Two apoptotic critical factors—lowering of the mitochondrial transmembrane potential ($\Delta\Psi_m$) and activation of caspase 3/7 —were significantly increased after 1 hour of sevoflurane treatment. Apoptosis and necrosis of PMN were determined by comet assay and flow cytometric analysis of annexin V-FITV protein binding to the cell surface and propidium iodide staining. Exposure of PMN to sevoflurane markedly increased apoptosis in a dose-dependent manner. However, the necrotic effect induced by sevoflurane was not significant. In summary, these results are important for demonstrating the oxidative damage on sevoflurane-treated human PMN. This information will suggest whether the perioperative application of antioxidant to prevent the sevoflurane-induced oxidative cellular damage in surgical patient is indicated.

1. Introduction

Sevoflurane is an inhalation anesthetic used for general anesthesia and is part of the "flurane" family of anesthetics that includes isoflurane, desflurane, and enflurane (Burrows et al., 2004). It is applied by vaporization and typically given coexistent with other anesthetics to carry out the preferred level of anesthesia (Malviya et al., 2004). Recently, several studies have shown that ROS exist in sevoflurane-induced cardioprotection (de Ruijter et al., 2003; Bouwman et al., 2004). Moreover, sevoflurane can directly trigger the formation of peroxynitrite, inducing nitrolysation of sarcolemmal proteins (Bouwman et al., 2004). Other reports also demonstrated that sevoflurane exposure induces an increase of ROS, results in attenuation on mitochondrial electron transport as a fire of anesthetic preconditioning in fresh guinea pig heart experimental model (Riess et al., 2004; Riess et al., 2005).

The role of ROS has been implicated to be the causative factor in many human degenerative diseases of aging (Balaban et al., 2005; Stadtman et al., 2005). Many clinical conditions, such as atherosclerosis (Kovacic and Thurn, 2005; van Oostrom et al., 2005), carcinogenesis (Valko et al., 2004), ischemia/reperfusion damage (Szocs, 2004), cataractogenesis (Spector, 1995), neurodegenerative disorders (Rego and Oliveira, 2003), and rheumatoid arthritis (Hadjigogos, 2003) are related with ROS production. Furthermore, ROS also exist in inflammatory periods (Lin et al., 2005). The oxidative stress induces by increasing ROS is believe to aversely affect the state of normal human health.

Sevoflurane is an inhalation anesthetic and is widely used in anesthesia practice. However, it will distribute to blood stream and affect leukocytes. Up to now, information pertinent to the effect of sevoflurane on oxidative stress in human peripheral leukocytes is scant. In peripheral leukocytes, the polymorphonuclear neutrophils (PMN) are the major group that defeat pathogenic microorganisms by phagocytosis and generate extremely high amounts of ROS (Amer and Fibach, 2005). Many proinflammatory substances and chemical stimulants can provoke the production of ROS in PMN (Braga et al., 2003; Tripathy et al., 2003; Kopprasch et al., 2004). For that reason, to evaluate oxidative stress and cellular oxidative injuries in sevoflurane treated PMN, we designed to investigate those factors of oxidative stress including intracellular hydrogen peroxide, superoxide and nitric oxide (NO) production, intracellular glutathione (GSH) content, lowering of the mitochondrial transmembrane potential ($\Delta\Psi_m$), activation of caspase 3/7 and oxidative related apoptosis and necrosis.

2. Materials and Methods

2.1. Reagents

The hydroethidine (HE), chloromethylflourescein diacetate (CMF-DA) utilized herein was acquired from Molecular Probes, Inc. (Eugene, OR, USA). The Apo-one™ homogeneous caspase-3/7 assay kit was purchased from Promega Company (Madison, WI, USA). Annexin-V-FLUOS staining kit was obtained from Roche Applied Science Company (Penzberg, Germany). Propidium iodide (PI), 2',7'-dichlorodihydrofluorescein-diacetate (DCFH-DA), 4,5-diaminofluorescein (DAF-2), S-nitroso-N-acetylpenicillamine (SNAP), catalase, mannitol, superoxide dismutase (SOD), dexamethasone, N-acetylcysteine (NAC), trolox, carbonyl cyanide m-chlorophenylhydrazone (CCCP), Dulbecco's modified Eagle's medium (DMEM), and other chemicals were bought from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. PMN preparations and treatments

Peripheral whole blood was obtained from volunteers (25 ± 5 years), none of whom suffering from any known acute or chronic disease, showing any symptoms of infection or inflammation, nor taking any drugs recognized to influence the immune system. They had body mass indices within the normal ranges (20 to 25 Kg/m^2), and did not use a specific dietary regimen nor take antioxidant supplement. Peripheral whole blood samples were collected into vacutainer tubes containing EDTA. Red blood cells were hemolyzed using ammonium chloride lysing solution, (150 mM M NH_4Cl, 10 nM $KHCO_3$, 1 nM Na_4EDTA, pH 7.4). Leukocytes were then washed twice with incubation buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 10 mM Hepes, pH 7.4), and then incubated in the same buffer with various concentrations of sevoflurane and other drugs. The population of PMN was recognized using the flow cytometry gating (see Fig. 1). For the comet assay, 5 ml of whole blood were stratified on a discontinuous Ficoll-Hypaque density gradient (HistopaqueR 1077 and 1119, Sigma-Aldrich) and centrifuged (700 g, 30 min, 20^0C) to obtain PMN. PMN were then collected on the corresponding layer (1.077<density<1.119).

2.3. Flow cytometry analysis of H_2O_2 and/or peroxide content in PMN

PMN (10^6 cells/ml) were preincubated with 20 μM of DCFH-DA in the 5%CO_2 incubator for 15 minutes. DCFH-DA is freely permeable to cellular membrane. In cytoplasm, esterases catalyze DCFH-DA to DCFH, which is then oxidized by H_2O_2 and/or peroxide into dichlorofluorescein (DCF) and emits a bright green fluorescence. PMN were then stimulated with 1% and 3% of sevoflurane for 1 h at 37^0C in CO_2 incubator. In order to check the experimental system, we used 2 mM of H_2O_2 to treat PMN for 10 minutes as the positive control group. H_2O_2 is freely permeable to the cellular membrane and into cytoplasm. In cytoplasm, H_2O_2 directly converts DCFH-DA to DCF. For antioxidants and NO inhibitor studies, PMN was first pretreated with either one of catalase (200 U/ml), mannitol (50 mM), SOD (10 U/ml), dexamethasone (10 μM), NAC (10 mM), or trolox (50 μM) for 15 minutes, followed by preincubation with 20 μM of DCFH-DA for another 15 minutes, and finally treated with 3% of sevoflurane for 1 h. After drugs treatment, PMN were washed with incubation buffer, collected by centrifugation, suspended in the same buffer and analyzed by flow cytometry.

2.4. Flow cytometry analysis of superoxide content in PMN

PMN (10^6 cells/ml) were preincubated with 10 μM of HE in the 5% CO_2 incubator for 15 minutes. HE is freely permeable to cellular membrane, and after being oxidized by superoxide, it turns into ethidium and emits a bright red fluorescence. PMN were then stimulated with 1% and 3% of sevoflurane for 1 h at 37^0C in the CO_2 incubator. In order to check the experimental system, we used 50 μM of menadione to treat PMN for 1 h as the positive control group. The intracellular NAD(P)H oxidase can react with menadione to generate superoxide. After drugs treatment, PMN were washed with incubation buffer, collected by centrifugation, suspended in the same buffer and analyzed by flow cytometry.

2.5. Flow cytometry analysis of NO content in PMN

PMN (10^6 cells/ml) were preincubated with 1 μM of DAF-2 in the 5% CO_2 incubator for 15

minutes. PMN were then stimulated with 1% and 3% of sevoflurane for 1 h at 37^0C in the CO_2 incubator. In order to check the experimental system, we used 300 μM of SNAP, a NO donor to treat PMN for 1 h as the positive control group. After drugs treatment, PMN were washed with incubation buffer, collected by centrifugation, suspended in the same buffer and analyzed by flow cytometry.

2.6. Flow cytometry analysis of GSH content in PMN

PMN (10^6 cells/ml) were incubated with 2 mM H_2O_2, 1% and 3% of sevoflurane in the 5% CO_2 incubator for 1 h. The 2 mM H_2O_2-treated PMN were used as the GSH depletion group. After drugs treatment, the PMN were incubated with 25 μM of CMF-DA for 20 minutes at 37^0C in the CO_2 incubator. CMF-DA is colorless and nonfluorescent containing a mildly thiol reactive chloromethyl reactive group. This probe is primarily conjugated to the abundant tripeptide GSH by glutathione S-transferase. Once inside the cell, cytosolic esterases cleavage their acetates and then the chloromethyl group reacts with intracellular thiols, transforming the probe into a cell-impermeant fluorescent dye-thioether adduct, chloromethylflourescein (CMF). After CMF-DA staining, PMN were washed with incubation buffer, collected by centrifugation, suspended in the same buffer and analyzed by flow cytometry.

2.7. Flow cytometry analysis of mitochondrial transmembrane potential ($\Delta\Psi_m$) in PMN

PMN (10^6 cells/ml) were preincubated with 5 μM of rhodamine 123 in the 5% CO_2 incubator for 15 minutes. PMN were then stimulated with 1% and 3% of sevoflurane for 0.5 and 1 h at 37^0C in the CO_2 incubator. In order to check the experimental system, we used 1 μM of CCCP to treat PMN for 0.5 and 1 h as the $\Delta\Psi_m$ disrupt group. For $\Delta\Psi_m$ and ROS related studies, 1 μM of CCCP was used to pretreat PMN for 45 minutes, preincubated with 20 μM of DCFH-DA for another 15 minutes, and then treated with 3% of sevoflurane for 1 h. After drugs treatment, PMN were washed with incubation buffer, collected by centrifugation, suspended in the same buffer and analyzed by flow cytometry.

2.8. Flow cytometry analysis of caspase 3/7 activies in PMN

PMN (10^6 cells/ml) were preincubated with 1% or 3% of sevoflurane in the 5% CO_2 incubator for 1 h. The substrate, Z-DEVD-R110 for caspase 3/7, was diluted with a buffer to make the desired amount of homogeneous substrate reagent. After drugs treatment, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in a DMEM medium. Then the homogeneous substrate reagent was added to the cells, maintaining a 1:1 ratio of reagent to cell solution. After 2 h of incubation at 37^oC, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in incubation buffer. Substrate cleavage to release free R110 fluorescence intensity was measured by flow cytometry.

2.9. Flow cytometry analysis of apoptosis and necrosis in PMN

PMN (10^6 cells/ml) in DMEM serum-free medium were incubated with 2 mM H_2O_2, and 1% or 3% of sevoflurane in the 5% CO_2 incubator for 4 h. After drugs treatment, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in an annexin V-FITC-propidium iodide reactive solution. In early stage of apoptosis, one of the plasma membrane alternations is the translocation of phosphatidylserine (PS) from the inner membrane to the outer membrane. The analysis of PS on the outer membrane of apoptotic cell membrane is performed by using annexin-V-FITC and propidium iodide for the differentiation from necrotic cells. After 15 minutes of annexin V-FITC-propidium iodide incubation at room temperature, the PMN were washed once with incubation buffer, collected by centrifugation, suspended in incubation buffer. The percentage of apoptotic and necrotic PMN was measured by flow cytometry.

2.10. Comet assay (single-cell gel electrophoresis) in PMN

The comet assay was performed following the method of Chen *et al.* (2003). Isolated PMN was treated with 3% sevoflurane in the 5% CO_2 incubator for 1 and 1.5 h. Eighty-five μl of a mixture of 0.5% normal melting point agarose (NMP) and 0.5% low melting point agarose (LMP) in PBS (pH

7.4), was used to precoat the conventional microscopic slide and let dry on a flat surface at room temperature. Ten μl of cell suspension (1x 10^6 cells/ml) were gently mixed with 75 μl of 0.5% (w/v) LMP in PBS (pH 7.4). Seventy-five μl of this suspension was rapidly layered onto the above mentioned precoated-slides and covered with a coverslip. The slides stood at 4^0C for 5 min, the coverslip was removed, and slides were immersed in a freshly made lysis solution (2.5 M of NaCl, 100 mM Na2EDTA, 10 mM Tris and 1% (v/v) of Triton X-100 at pH 10) at 4^0C for 10 minutes. The slides were then put in a double row in a 260-mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na2EDTA for 10 min. Thereafter, electrophoresis (30 V, 300 mA) was conducted for 15 min at 4^0C. After electrophoresis, the slides were bathed in the cold neutralizing buffer (400 mM of Tris buffer, pH 7.5) at 4^0C for 10 min. Slides were then placed into methanol for 5 min to dehydrate the cells, and stored in a low humidity environment before silver staining. Silver staining was performed following the method of Reinhardt-Poulin *et al.* (2000). One hundred comets on each slide were counted and scored visually according to the relative length of the tail, and were assigned as class 0 (undamaged) to class 4 (severely damaged). Thus, the total score for each slide could range from 0 to 400 because 100 cells were observed individually in each comet assay.

2.11. Statistical Analysis

Data is presented as means $\pm$ standard deviation (SD) and analyzed using one-way ANOVA with Scheffe's test. A *p* value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Sevoflurane induced intracellular H_2O_2 and/or peroxide in PMN

To evaluate the oxidative stress of sevoflurane in PMN, we first tested the production of intracellular H_2O_2 and/or peroxide at the concentration of 0%, 1%, and 3% of sevoflurane for 1 h by flow cytometry and DCFH-DA staining (Hsieh et al., 2004). In this experimental model, 2 mM H_2O_2 was added to PMN for 5 minutes as H_2O_2-positive groups. The leukocytes were sorted as 3 groups, including lymphocytes (R1), monocytes (R2), and polymorphonucleocytes (R3) by the CellQuest software (Fig. 1). The R3 region was gated and evaluated in all of present studies. The DCF fluorescence intensity expresses the related intracellular H_2O_2 and/or peroxide level. The DCF fluorescence was markedly increased to 7702.6±923.2 in H_2O_2-positive groups (Fig. 2). As shown in Fig. 2, the DCF fluorescence increased significantly in PMN after 1% and 3% of sevoflurane treatment as compared with untreated PMN. Sevoflurane exhibited 757.3±123.6 and 768.8±108.3 of DCF fluorescence at 1% and 3%, respectively.

The intracellular superoxide level was assayed by hydroethidine (HE) staining in sevoflurane-treated PMN after 30 minutes treatment. Fifty μM of menadione treatment for 30 minutes was carried out as intracellular superoxide-positive control groups (Niemczyk et al., 2004). As shown in Fig. 3, 50 μM of menadione markedly increased the HE fluorescence in PMN as compared with untreated PMN. The HE fluorescence increased from 515.9±146.3 to 858.5±66.7. In sevoflurane-treated PMN, the HE fluorescence significantly increased to 837.2±160.0 and 1078.1±19.9 in 1% and 3% treatment, respectively.

3.2. Sevoflurane induced intracellular NO in PMN

Nitric oxide (NO) has many important biologic functions, but it produces many damaging effects on several biological molecular, such as lipids, proteins and DNA through switch into reactive nitrogen oxide species including nitrogen dioxide, dinitrogen trioxide, and peroxynitrite (Kikugawa et al., 2005). To investigate whether NO appears during sevoflurane treatment, we examined the production of NO by DAF-2 staining, a NO specific probe and flow cytometry (Hsieh et al., 2005). SNAP, a NO donor was used as an intracellular NO-positive control group (Kwon et al., 2005). The intracellular NO level expressed by DAF-2 fluorescence are shown in Fig. 4. Compared with the untreated PMN, the PMN demonstrated a significant increase in DAF-2 fluorescence (631.6±29.8) after exposure to 300 μM SNAP for 30 minutes, as expected. The DAF-2 fluorescence value was 334.4±29.2 in the untreated PMN versus 308.4±15.5 and 387.4±13.5 in 1% and 3% sevoflurane-treated PMN, respectively.

3.3. Sevoflurane induced GSH depletion in PMN

Many studies have reported that GSH depletion is a key factor on chromosomal DNA fragmentation and apoptosis or necrosis in oxidative stress-induced cell death (Higuchi, 2004). Our results in the present study have demonstrated that sevoflurane could increase intracellular ROS and NO level in PMN. The effect of sevoflurane on intracellular GSH depletion is thus worth investigating. In our previously published studies, the intracellular GSH level was analyzed by CMF-DA staining, a GSH specific probe and flow cytometry (Hsieh et al., 2004). The CMF fluorescence is directly related to intracellular GSH level. In this present study, treatment with 2 mM H_2O_2 for 1 h was used as positive control group to induce intracellular GSH depletion. As shown in Fig 5, the CMF fluorescence value in untreated PMN was 1789.8 ± 23.5. The CMF fluorescence in H_2O_2-treated PMN was markedly decreased to 861.4 ± 38.1. In the meantime, sevoflurane treatment for 1 h also significant reduced the CMF fluorescence to 1178.3 ± 43.5 and 1114.7 ± 50.6 in 1% and 3% treatment, respectively. It means that sevoflurane promotes intracellular GSH depletion in PMN.

3.4. Chief ROS species and the preventive effect of antioxidants and NO inhibitor on sevoflurane treatment in PMN

In order to determine what is the chief component of ROS generation and the preventive effect of antioxidants and NO inhibitor on sevoflurane treatment, the effect of several ROS scavengers, such as catalase, mannitol, superoxide dismutase (SOD); antioxidants such as N-acetylcysteine (NAC) and trolox; and dexamethasone, a NO inhibitor, on sevoflurane-induced intracellular DCF fluorescence in PMN cells were determined. As shown in Fig. 6, the SOD failed to ameliorate the increased DCF fluorescence in sevoflurane-treated PMN; on the contrary, it prompted the DCF fluorescence accumulation in sevoflurane-treated PMN. Catalase, a H_2O_2 scavenger, mannitol, a hydroxyl radiacal scavenger, and dexamethasone could significantly decrease intracellular DCF fluorescence in sevoflurane-treated PMN. The DCF-fluorescence-diminishing-effect also existed for both antioxidants, NAC and trolox. It is interesting to note that the lowest intracellular DCF fluorescence appeared in catalase-treated PMN, thereby suggesting that the chief ROS specie producing by sevoflurane is H_2O_2.

3.5. Effect of mitochondrial transmembrane potential ($\Delta\Psi_m$) in sevoflurane-treated PMN

Experimental data from other studies have demonstrated that ROS can disrupt $\Delta\Psi_m$ (Derouet-Humbert et al., 2005; Tay et al., 2005). Because sevoflurane induced a large amount of ROS in PMN, we further studied the $\Delta\Psi_m$ in sevoflurane-treated PMN. Using rhodamine 123 staining, the $\Delta\Psi_m$ was evaluated in sevoflurane-treated PMN. In the mean time, the CCCP (1 μM) was used as a positive control to induce decreasing of $\Delta\Psi_m$. As expect, CCCP resulted in marked $\Delta\Psi_m$ decrease in PMN at 0.5 and 1 h (Fig. 7A). It is interesting to note that 3% of sevoflurane resulted significantly increasing of $\Delta\Psi_m$ in PMN after 0.5 h treatment as compared with their untreated cells. The rhodamine 123 fluorescence in untreated and 3% sevoflurane-treated PMN displayed 657.0 ± 19.9 and 897.8 ± 83.7, respectively. The decrease in $\Delta\Psi_m$ was also noted in sevoflurane-treated PMN after 1 h treatment (Fig. 7A). The rhodamine 123 fluorescence was significantly decreased to 429.1 ± 68.4 and 427.6 ± 80.1 in 1% and 3% of sevoflurane, respectively, as compared with 616.7 ± 23.2 in untreated PMN.

The increase of $\Delta\Psi_m$ in PMN after sevoflurane treatment at 0.5 h is a novel phenomenon. It is the first discovery in the field of anesthetic practice. Some studies pointed out that the increase of $\Delta\Psi_m$ is related to ROS generation (Chen et al., 2005; Lee et al., 2005). We further studied the relation between $\Delta\Psi_m$ and intracellular ROS production in sevoflurane treatment. On this basis, we examined whether CCCP, a distinct $\Delta\Psi_m$ inhibitor, could block the increasing of $\Delta\Psi_m$ therefore diminish the intracellular ROS by DCF fluouoscence in sevoflurane-treated PMN. For this purpose, the PMN were subdivided into four different groups for treatment: (a) untreated; (b) 3% sevoflurane alone; (c) CCCP and 3% sevoflurane; (d) CCCP alone, respectively. In group (c), following pre-treatment with 1 μM CCCP for 1 h, PMN were subjected to 3% sevoflurane treatment for another 1 h. As shown in Fig. 7B, there were no significant different on intracellular DCF fluorescence between untreated and CCCP-treated PMN (502.2 ± 8.5 v.s. 472.2 ± 16.4). However, 3% of sevoflurane increased the DCF fluorescence to 651.4 ± 75.6. The pre-treatment of

CCCP could not abolish the sevoflurane-triggered ROS increase in PMN, it exhibited 652.5±83.1 of DCF fluorescence.

3.6. Sevoflurane increased caspase 3/7 activities in PMN cells

Caspases are crucial factors of apoptosis. Among them, caspase 3/7 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins (Porter and Jänicke, 1999). Some reports have demonstrated ROS or NO induce apoptosis via caspase 3/7 activation (Rolye et al., 2004; Chen et al., 2005). To determine the role of caspase 3/7 activation in sevoflurane-induced oxidative stress, we measured the activities of caspase 3/7 in sevoflurane treated PMN. Activities of caspase 3/7 during sevoflurane-induced oxidative stress were determined by the proteolytic activity with a fluorogenic Ac-DEVD-R110 substrate. Activated caspase 3/7 can cleavage the non-fluorescence molecular, Ac-DEVD-R110, then release fluorescence molecule R110 in cells. The intensity of R110 fluorescence exhibits the caspase 3/7 activities. As shown in Fig. 8, treatment with 1% and 3% of sevoflurane resulted in significant increased in activities of caspase 3/7 for 1 h as compared with untreated PMN. These data indicated that activation of the caspase 3/7 participated in sevoflurane-induced oxidative stress.

3.7. Sevoflurane induced apoptosis in PMN cells

We further hypothesized that sevoflurane would sensitize PMN to activation of apoptosis. Apoptosis and necrosis of PMN was determined by flow cytometric analysis of annexin V-FITC binding to the cell surface and propidium iodide staining. The percentage of apoptotic and necrotic cells after 1% and 3% of sevoflurane treatment for 2 h was evaluated by dual color flow cytometric analysis. PMN cells were assessed and representative data are shown in Fig. 9A. The percentage of apoptotic cells increased from 1.95% to 6.01% when treated with 1% sevoflurane. Treatment of cells with 3% of sevoflurane dramatically increased the percentage of apoptosis to 10.08% as well. Results in Fig. 9A also show that sevoflurane could not induce obvious necrosis in PMN: the percentage of necrotic cells was less than 2%. Fig. 9B summarizes the results of DNA damage score in human PMN treated with 3% sevoflurane for 1 and 1.5 h, as measured by comet assay. Treatment with sevoflurane exhibited significant DNA damage score of 165±5 and 196±9 at 1 and 1.5 h, respectively, when compared to that of the untreated group (83±4).

4. Discussion

In this study we have demonstrated that sevoflurane, at a clinically attainable concentration, induces cellular damage in normal peripheral PMN by enhancing the generation of ROS or NO, inducing GSH depletion, caspase 3/7 activation, and decreasing $\Delta\Psi_m$. The major role of PMN is to defeat pathogenic microorganisms after phagocytosis by generating ROS and toxic molecules (Walrand et al., 2005). However, PMN produce enough quantities of ROS during an oxidative burst to be autotoxic and harmful to their own functions and to probably result in DNA damage, protein and lipid oxidation and cell membrane destruction (Walrand et al., 2005). Our studies found that the level of intracellular ROS generation by sevoflurane stimulation in PMN was larger than in lymphocytes and monocytes (data not shown). For these reasons, we focus the present studies on PMN to evaluate various events of cellular damage during sevoflurane treatment.

ROS have become known as major signaling molecules in the modulation of various cellular processes. Immoderate generation of ROS may result in attack of many diseases and harm to most intracellular and extracellular biomolecules in a living organism. Moreover, ROS can directly prompt and/or adjust apoptosis and necrosis (Canakci et al., 2005). Mitochondria is a major producer of ROS, which mainly consist of superoxide anion, hydrogen peroxide, and hydroxyl radical (Larochette et al., 1999; Brand and Murphy, 1987). The main source of ROS in most cell types is possibly the electron leakage from mitochondrial electron transport chain that diminishes molecular oxygen to superoxide anion. Superoxide dismutase (SOD) will convert superoxide anion to hydrogen peroxide and then produce hydroxyl radical rapidly via either Fenton reaction (Hsieh et al., 2004) or Harber-Weiss reaction (Hsieh et al., 2004). Our results found that intracellular superoxide level was increased about 2-fold at 30 minutes (Fig. 3) but not at 1 h (data not shown) in sevoflurane-treated PMN. In addition, our data also demonstrated that pretreatment with SOD can significantly increase the DCF fluorescence in sevoflurane-treated

PMN (Fig. 6). These phenomena explain that the superoxide induced by sevofluane may be rapidly converted by intracellular SOD to hydrogen peroxide, and then reacted with non-fluorescence of DCFH to a fluorescence substance, DCF at later time (1 h) (Fig. 2). NO is known to modulate various function of PMN such as chemotaxis, adherence, aggregation, and generation of ROS (Sharma et al., 2004). Our results demonstrated that dexamethasone, a NO inhibitor could significantly inhibit sevoflurane-induced intracellular DCF fluorescence (Fig. 6). We propose that part of intracellular ROS induced by sevoflurane is coming from NO generation.

The $\Delta\Psi_m$ reflects the energy accumulated in the electrochemical gradient across the inner mitochondrial membrane, which in turn is used by F0F1-ATPase to change adenosine 5'-diphosphate to ATP throughout oxidative phosphorylation. Mitochondrial hyperpolarization and transient ATP depletion represent early and reversible steps in T-cell activation and apoptosis (Perl et al., 2004). It is interesting to find that $\Delta\Psi_m$ exhibited transient increase at 0.5 h in 3% sevoflurane-treated PMN (Fig 7A). The $\Delta\Psi_m$ increase often implies mitochondrial hyperpolarization. Several studies demonstrated that mitochondrial hyperpolarization induced by some substances, such as lysophophatidylcholine, linoleic acid or farnesol usually accompanies with ROS overproduction (Machida and Tanaka, 1999; Lee et al., 2005). These phenomena excited us to discover the relationship between ROS production and transient mitochondrial hyperpolarization in sevoflurane-treated PMN. Using CCCP pretreatment to inhibit the mitochondrial hyperpolarization, we evaluated the intracellular DCF fluorescence by flow cytometry in sevoflurane-treated PMN. Our results found that the increase of intracellular DCF fluorescence by sevoflurane treatment could not be abolished by CCCP in PMN (Fig. 7B). These results suggest that the ROS increase in sevoflurane-treated PMN does not relate to transient mitochondrial hyperpolarization.

Kanna et al. (2002) demonstrated that sevoflurane shows to excite the plasmalemmal Ca^{2+} influx and thereby raise the pulmonary arterial valvular endothelial intracellular calcium level. Other studies also showed that the intracellular ROS generation is accompanied with an increase of the intracellular calcium in cardiac cells and leukocytes by low energy visible light and toxic substance stresses, respectively. (Heiskanen et al., 1995; Lavi et al., 2003). On the other hand, isoflurane applied during ischemia enhances intracellular calcium accumulation in ventricular myocytes in part by reactive oxygen species (Dworschak et al., 2002). These studies explain that a relative event exists between ROS generation and intracellular calcium accumulation. Our results found that sevoflurane could not induce significant ROS increase in PMN by means of calcium-free PBS buffer experimental system (data not shown), by contrast, the ROS production was significantly increased in sevoflurane-treated PMN with calcium-containing buffer system. These results explain that the intracellular ROS generation induced by sevoflurane is partially dependent on intracellular calcium level.

Cells contain several antioxidant systems to protect the injury induced by increased intracellular ROS. Among them, GSH is an antioxidant that it defends cells from ROS-induced apoptosis (Yu, 1994). Many studies point out the GSH depletion is an importance factor that increases percentage of cells undergoing apoptosis; on other contrary, NAC, a GSH precursor, decreases apoptosis induced by GSH depletion (Beaver and Waring, 1995; Kito et al., 2002). Other reports demonstrated that caspase 3/7 activation is an important step in glutathione depletion-induced apoptosis in resting and inflammatory neutrophils (O'Neill et al., 2000). In our present study, we observed that sevoflurane produced intracellular GSH depletion in PMN. By contrast, pretreatment of NAC significantly protected PMN subject to sevoflurane-induced intracellular DCF fluorescence (Fig. 6). We proposed that intracellular GSH depletion in PMN may be an important factor for sevoflurane-induced apoptosis to occur, and afterward activation of cellular caspase 3/7 appears to be a significance mechanism. With the above data, we suggest that appropriate antioxidants may be needed during the anesthetic periods when using sevoflurane.

References

Amer, J., Fibach, E., 2005. Chronic oxidative stress reduces the respiratory burst response of neutrophils from beta-thalassaemia patients. Br. J. Haematol. 129, 435-441.

Balaban, R.S., Nemoto, S., Finkel, T., 2005. Mitochondria, oxidants, and aging. Cell 120, 483-495.

Beaver, J.P., Waring, P. A., 1995. Decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. Eur. J. Biol. 68, 47-54.

Bouwman, R.A., Musters, R.J., van Beek-Harmsen, B.J., de Lange, J.J., Boer, C., 2004. Reactive oxygen species precede protein kinase C-delta activation independent of adenosine triphosphate-sensitive mitochondrial channel opening in sevoflurane-induced cardioprotection. Anesthesiology 100, 506-514.

Braga, P.C., dal Sasso, M., Bovio, C., Sgaragli, G.P., 2003. Effect of butylated hydroxyanisole and some of its derivatives on human neutrophil oxidative burst: chemiluminescence evaluation. Pharmacology 68, 9-16.

Brand, M.D., Murphy, M.P., 1987. Control of electron flux through the respiratory chain in mitochondria and cells. Biol. Rev. Camb. Philos. Soc., 62,141-193.

Burrows, D.L., Nicolaides, A., Stephens, G.C., Ferslew, K.E., 2004. The distribution of sevoflurane in a sevoflurane induced death. J Forensic Sci. 49, 1-4.

Canakci, C.F., Cicek, Y., Canakci, V., 2005. Reactive oxygen species and human inflammatory periodontal diseases. Biochemistry (Mosc). 70, 619-628.

Chen, S.C., Kao, C.M., Huang, M.H., Shih, M.K., Chen, Y.L., Huang, S.P., Liu, T.Z., 2003. Assessment of genotoxicity of benzidine and its structural analogues to human lymphocytes using comet assay. Toxicol. Sci. 72, 283-288.

Chen, Z.H., Na, H.K., Hurh, Y.J., Surh, Y.J., 2005. 4-Hydroxyestradiol induces oxidative stress and apoptosis in human mammary epithelial cells: possible protection by NF-kappaB and ERK/MAPK. Toxicol. Appl. Pharmacol. 208, 46-56.

Derouet-Humbert, E., Roemer, K., Bureik, M., 2005. Adrenodoxin (Adx) and CYP11A1 (P450scc) induce apoptosis by the generation of reactive oxygen species in mitochondria. Biol. Chem. 386, 453-461.

Dworschak, M., Breukelmann, D., Hannon, J.D., 2004. Isoflurane applied during ischemia enhances intracellular calcium accumulation in ventricular myocytes in part by reactive oxygen species. Acta. Anaesthesiol. Scand. 48, 716-721.

Hadjigogos, K., 2003. The role of free radicals in the pathogenesis of rheumatoid arthritis. Panminerva Med. 45, 7-13.

Heiskanen, K.M., Naarala, J., Savolainen, K.M., 1995. The effects of N-(5-vinyl-1,3-thiazolidin-2-ylidene)phenylamine (5-VTPA) on the changes in free intracellular calcium and the production of reactive oxygen metabolites in human leukocytes. Toxicology 100, 195-202.

Higuchi, Y., 2004. Glutathione depletion-induced chromosomal DNA fragmentation associated with apoptosis and necrosis. J. Cell. Mol. Med. 8, 455-464.

Hsieh, T.J., Liu, T.Z., Chern, C.L., Tsao, D.A., Lu, F.J., Syu, Y.H., Hsieh, P.Y., Hu, H.S., Chang, T.T., Chen, C.H., 2005. Liriodenine inhibits the proliferation of human hepatoma cell lines by blocking cell cycle progression and nitric oxide-mediated activation of p53 expression. Food Chem. Toxicol. 43, 1117-1126.

Hsieh, T.J., Liu, T.Z., Chia, Y.C., Chern, C.L., Lu, F.J., Chuang, M.C., Mau, S.Y., Chen, S.H., Syu, Y.H., Chen, C.H., 2004. Protective effect of methyl gallate from *Toona sinensis* (Meliaceae) against hydrogen peroxide-induced oxidative stress and DNA damage in MDCK cells. Food Chem. Toxicol. 42, 843-850.

Kanna, T., Akata, T., Izumi, K., Nakashima, M., Yonemitsu, Y., Hashizume, M., Takahashi, S., 2002. Sevoflurane and bradykinin-induced calcium mobilization in pulmonary arterial valvular endothelial cells in situ: sevoflurane stimulates plasmalemmal calcium influx into endothelial cells. J. Cardiovasc. Pharmacol. 40, 714-724.

Kikugawa, K., Oikawa, N., Miyazawa, A., Shindo, K., Kato, T., 2005. Interaction of nitric oxide with glutathione or cysteine generates reactive oxygen species causing DNA single strand breaks. Biol. Pharm. Bull. 28, 998-1003.

Kito, M., Akao, Y., Ohishi, N., Yagi, K., Nozawa, Y., 2002. Arsenic trioxide-induced apoptosis and its enhancement by buthionine sulfoximine in hepatocellular carcinoma cell lines. Biochem. Biophys. Res. Commun. 291, 861-867.

Kopprasch, S., Pietzsch, J., Graessler, J., 2004. The protective effects of HDL and its constituents against neutrophil respiratory burst activation by hypochlorite-oxidized LDL. Mol. Cell.

Biochem. 258, 121-127.

Kovacic, P., Thurn, L.A., 2005. Cardiovascular toxicity from the perspective of oxidative stress, electron transfer, and prevention by antioxidants. Curr. Vasc. Pharmacol. 3, 107-117.

Kwon, K.B., Kim, E.K., Lim, J.G., Shin, B.C., Han, S.C., Song, B.K., Kim, K.S., Seo, E.A., Ryu, D.G., 2005. Protective effect of Coptidis Rhizoma on S-nitroso-N-acetylpenicillamine (SNAP)-induced apoptosis and necrosis in pancreatic RINm5F cells. Life Sci. 76, 917-929.

Larochette, N., Decaudin, D., Jacotot, E., Brenner, C., Marzo, I., Susin, S.A., Zamzami, N., Xie, Z., Reed, J., Kroemer, G., 1999. Arsenite induces apoptosis via a direct effect on mitochondrial permeability transition pore. Exp. Cell Res. 249, 413-421.

Lavi, R., Shainberg, A., Friedmann, H., Shneyvays, V., Rickover, O., Eichler, M., Kaplan, D., Lubart, R., 2003. Low energy visible light induces reactive oxygen species generation and stimulates an increase of intracellular calcium concentration in cardiac cells. J. Biol. Chem. 278, 40917-40922.

Lee, C.S., Kim, Y.J., Ko, H.H., Han, E.S., 2005. Synergistic effects of hydrogen peroxide and ethanol on cell viability loss in PC12 cells by increase in mitochondrial permeability transition. Biochem. Pharmacol. 70, 317-325.

Lin, Y., Berg, A.H., Iyengar, P., Lam, T.K., Giacca, A., Combs, T.P., Rajala, M.W., Du, X., Rollman, B., Li, W., Hawkins, M., Barzilai, N., Rhodes, C.J., Fantus, I.G., Brownlee, M., Scherer, P.E., 2005. The hyperglycemia-induced inflammatory response in adipocytes: the role of reactive oxygen species. J. Biol. Chem. 280, 4617-4626.

Machida, K., Tanaka, T., 1999. Farnesol-induced generation of reactive oxygen species dependent on mitochondrial transmembrane potential hyperpolarization mediated by F(0)F(1)-ATPase in yeast. FEBS Lett. 462, 108-112.

Malviya, S., Voepel-Lewis, T., Tait, A.,Reynolds, P.I., Gujar, S.K., Gebarski, S.S., Petter Eldevik, O., 2004. Pentobarbital vs choral hydrate for sedation of children undergoing MRI: efficacy and recovery characteristics. Paediatr. Anaesth.14, 589-595.

Niemczyk, E., Majczak, A., Hallmann, A., Kedzior, J., Wozniak, M., Wakabayashi, T., 2004. A possible involvement of plasma membrane NAD(P)H oxidase in the switch mechanism of the cell death mode from apoptosis to necrosis in menadione-induced cell injury. Acta. Biochim. Pol. 51, 1015-1022.

O'Neill, A.J., O'Neill, S., Hegarty, N.J., Coffey, R.N., Gibbons, N., Brady, H., Fitzpatrick, J.M., Watson, R.W., 2000. Glutathione depletion-induced neutrophil apoptosis is caspase 3 dependent. Shock. 14, 605-609.

van Oostrom, A.J., van Wijk, J., Cabezas, M.C., 2004. Lipaemia, inflammation and atherosclerosis: novel opportunities in the understanding and treatment of atherosclerosis. Drugs 64, 19-41.

Perl., A, Nagy, G., Gergely, P., Puskas, F., Qian, Y., Banki, K., 2004. Apoptosis and mitochondrial dysfunction in lymphocytes of patients with systemic lupus erythematosus. Methods Mol. Med. 102, 87-114.

Porter, A,G., Jänicke, R.U., 1999. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 6, 99-104.

Rego, A.C., Oliveira, C.R., 2003. Mitochondrial dysfunction and reactive oxygen species in excitotoxicity and apoptosis: implications for the pathogenesis of neurodegenerative diseases. Neurochem. Res. 28, 1563-1574.

Reinhardt-Poulin, P., McLean, J.R., Deslauriers, Y., Gorman, W., Cabat, S., Rouabhia, M., 2000. The use of silver-stained "comets" to visualize DNA damage and repair in normal and Xeroderma pigmentosum fibroblasts after exposure to simulated solar radiation. Photochem. Photobiol. 71, 422-425.

Riess, M.L., Eells, J.T., Kevin, L.G., Camara, A.K., Henry, M.M., Stowe, D.F., 2004. Attenuation of mitochondrial respiration by sevoflurane in isolated cardiac mitochondria is mediated in part by reactive oxygen species. Anesthesiology 100, 498-505.

Riess, M.L., Kevin, L.G., McCormick, J., Jiang, M.T., Rhodes, S.S., Stowe, D.F., 2005. Anesthetic preconditioning: the role of free radicals in sevoflurane-induced attenuation of mitochondrial electron transport in Guinea pig isolated hearts. Anesth. Analg. 100, 46-53.

de Ruijter, W., Musters, R.J., Boer, C., Stienen, G.J., Simonides, W.S., de Lange, J.J., 2003. The cardioprotective effect of sevoflurane depends on protein kinase C activation, opening of

mitochondrial K(+)(ATP) channels, and the production of reactive oxygen species. Anesth. Analg. 97, 1370-1376.

Sharma, P., Raghavan, S.A., Saini, R., Dikshit, M., 2004. Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide. J. Leukoc. Biol. 75, 1070-1078.

Spector, A., 1995. Oxidative stress-induced cataract: Mechanism of action. FASEB J. 9, 1173-1182.

Stadtman, E.R., Van Remmen, H., Richardson, A., Wehr, N.B., Levine, R.L., 2005. Methionine oxidation and aging. Biochim. Biophys. Acta. 1703, 135-140.

Szocs, K., 2004. Endothelial dysfunction and reactive oxygen species production in ischemia/reperfusion and nitrate tolerance. Gen. Physiol. Biophys.; 23: 265-295.

Tay, V.K., Wang, A.S., Leow, K.Y., Ong, M.M., Wong, K.P., Boelsterli, U.A., 2005. Mitochondrial permeability transition as a source of superoxide anion induced by the nitroaromatic drug nimesulide in vitro. Free Radic Biol Med. 39, 949-959.

Tripathy, D., Mohanty, P., Dhindsa, S., Syed, T., Ghanim, H., Aljada, A., Dandona, P., 2003. Elevation of free fatty acids induces inflammation and impairs vascular reactivity in healthy subjects. Diabetes 52, 2882-2887.

Valko, M., Izakovic, M., Mazur, M., Rhodes, C.J., Telser, J., 2004. Role of oxygen radicals in DNA damage and cancer incidence. Mol. Cell Biochem. 266, 37-56.

Walrand, S., Farges, M., Dehaese, O., Cardinault, N., Minet-Quinard, R., Grolier, P., Bouteloup-Demange, C., Ribalta, J., Winklhofer-Roob, B.M., Rock, E., Vasson, M.P., 2005. In vivo and in vitro evidences that carotenoids could modulate the neutrophil respiratory burst during dietary manipulation. Eur. J. Nutr. 44,114-120.

Yu, B.P., 1994. Cellular defenses against damage from reactive oxygen species. Physiol. Rev. 74, 139-162.

Figures and Legends
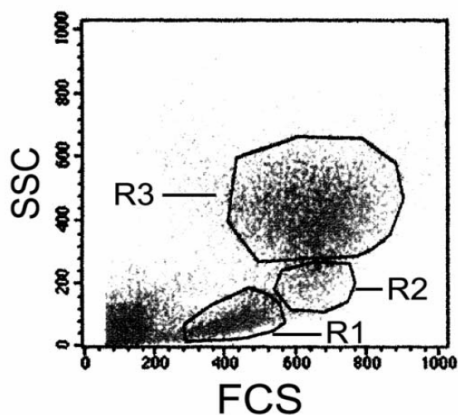
Fig. 1. Gating PMN by CellQuest software in flow cytometric analysis. Peripheral whole blood samples were collected into vacutainer tubes containing EDTA. Red blood cells were haemolyzed using ammonium chloride lysing solution (150 mM M NH_4Cl, 10 nM $KHCO_3$, 1 nM Na_4EDTA, pH 7.4). Leukocytes were then washed twice with incubation buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 10 mM Hepes, pH 7.4), and then incubated in the same buffer with various concentrations of sevoflurane and other drugs. After drugs treatment, the PMN were analysis by flow cytometry and CellQuest software. The representative plots depict forward scatter (FSC) on the x-axis and side scatter (SSC) on the y-axis. The leukocytes cells were sorted as 3 groups, including lymphocytes (R1), monocytes (R2), and PMN (R3) by the CellQuest software. The R3 region was gated and evaluated in all of present studies.
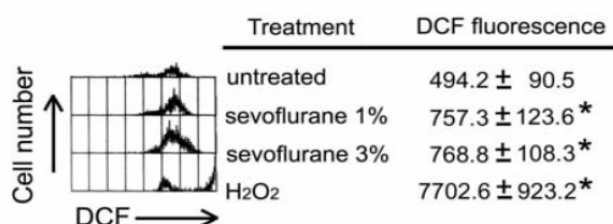

Treatment	DCF fluorescence
untreated	494.2 ± 90.5
sevoflurane 1%	757.3 ± 123.6 *
sevoflurane 3%	768.8 ± 108.3 *
H_2O_2	7702.6 ± 923.2 *

Fig. 2. Effect of sevoflurane on intracellular H_2O_2 and/or peroxide level in PMN. PMN (10^6 cells/ml) were preincubated in 5% CO_2 incubator for 15 minutes with 20 μM DCFH-DA. After DCFH-DA treatment, PMN were then stimulated with 1% and 3% sevoflurane for 1 h in 37^0C CO_2 incubator. For experimental positive control, PMN were treated with 2 mM H_2O_2 for 10 minutes. After drugs treatment, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The DCF fluorescence reflecting the level of intracellular H_2O_2 and/or peroxide in PMN was measured by flow cytometry. The data represents the DCF fluorescence intensity within the PMN. The values shown are mean±standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*).
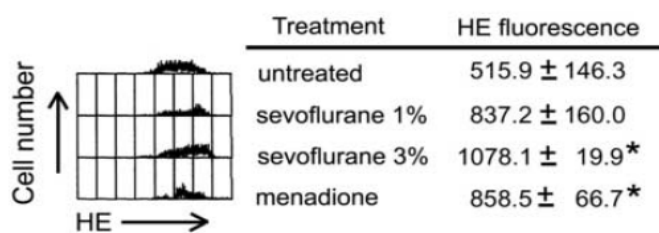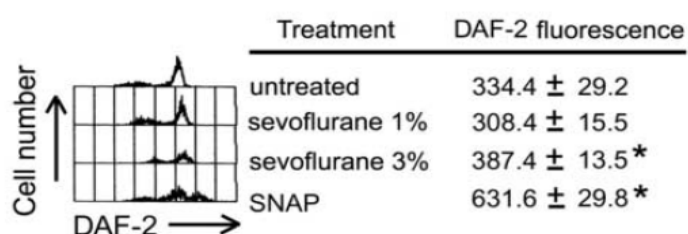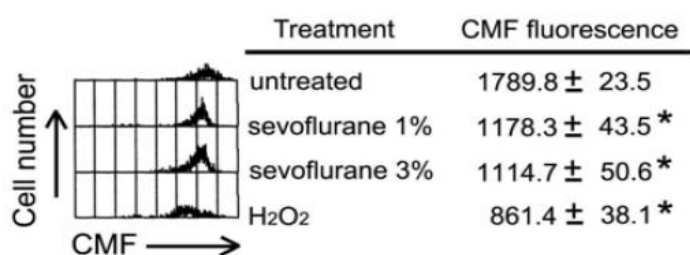
Treatment	HE fluorescence
untreated	515.9 ± 146.3
sevoflurane 1%	837.2 ± 160.0
sevoflurane 3%	1078.1 ± 19.9*
menadione	858.5 ± 66.7*

Fig. 3. Effect of sevoflurane on intracellular superoxide level in PMN. PMN (10^6 cells/ml) were preincubated in 5% CO_2 incubator for 15 minutes with 10 μM hydroethidine (HE). After HE treatment, PMN were then stimulated with 1% and 3% sevoflurane for 30 minutes in 37^0C CO_2 incubator. For experimental positive control, PMN were treated with 50 μM menadione for 30 minutes. After drugs treatment, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The HE fluorescence reflecting the level of intracellular superoxide in PMN was measured by flow cytometry. The data represents the HE fluorescence intensity within the PMN. The values shown are mean$\pm$ standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*).

Treatment	DAF-2 fluorescence
untreated	334.4 ± 29.2
sevoflurane 1%	308.4 ± 15.5
sevoflurane 3%	387.4 ± 13.5*
SNAP	631.6 ± 29.8*

Fig. 4. Effect of sevoflurane on intracellular NO level in PMN. PMN (10^6 cells/ml) were preincubated in 5% CO_2 incubator for 15 minutes with 1 μM 4,5-diaminofluorescein (DAF-2). After DAF-2 treatment, PMN were then stimulated with 1% and 3% sevoflurane for 30 minutes in 37^0C CO_2 incubator. For experimental positive control, PMN were treated with 300 μM SNAP for 30 minutes. After drugs treatment, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The DAF-2 fluorescence reflecting the level of intracellular NO in PMN was measured by flow cytometry. The data represents the DAF-2 fluorescence intensity within the PMN. The values shown are mean$\pm$standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p< 0.05$ (*).

Treatment	CMF fluorescence
untreated	1789.8 ± 23.5
sevoflurane 1%	1178.3 ± 43.5*
sevoflurane 3%	1114.7 ± 50.6*
H_2O_2	861.4 ± 38.1*

Fig. 5. Effect of sevoflurane on intracellular GSH content in PMN. PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator for 1 h with 1% and 3% sevoflurane. For GSH depletion group, PMN were treated with 2 mM H_2O_2 for 1 h. After drugs treatment, the PMN were incubated with 25 μM CMF-DA for 20 minutes in 37^0C CO_2 incubator. After CMF-DA staining, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The CMF fluorescence reflecting the related content of intracellular GSH in PMN was measured by flow cytometry. The data represents the CMF fluorescence intensity within the PMN. The values shown are mean$\pm$standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p< 0.05$ (*).
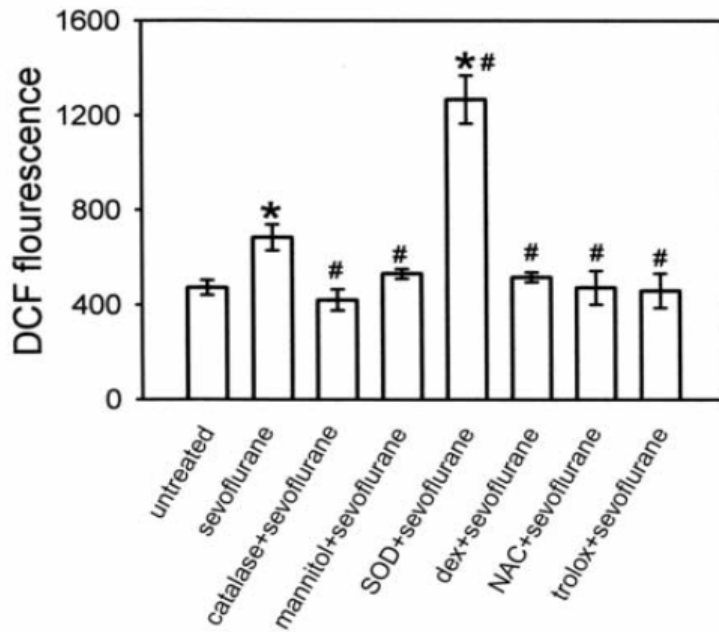
Fig. 6. The preventive effect of antioxidants and NO inhibitor on sevoflurane treatment in PMN. PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator with incubation buffer. Catalase (200 U/ml), mannitol (50 mM), SOD (10 U/ml), dexamethasone (10 μM), NAC (10 mM), and trolox (50 μM) were used to pretreat PMN for 15 minutes, the PMN were then preincubated with 20 μM DCFH-DA for another15 minutes, and finally treated with 3% sevoflurane for 1 h. After drugs treatment, PMN were washed once with incubation buffer, collected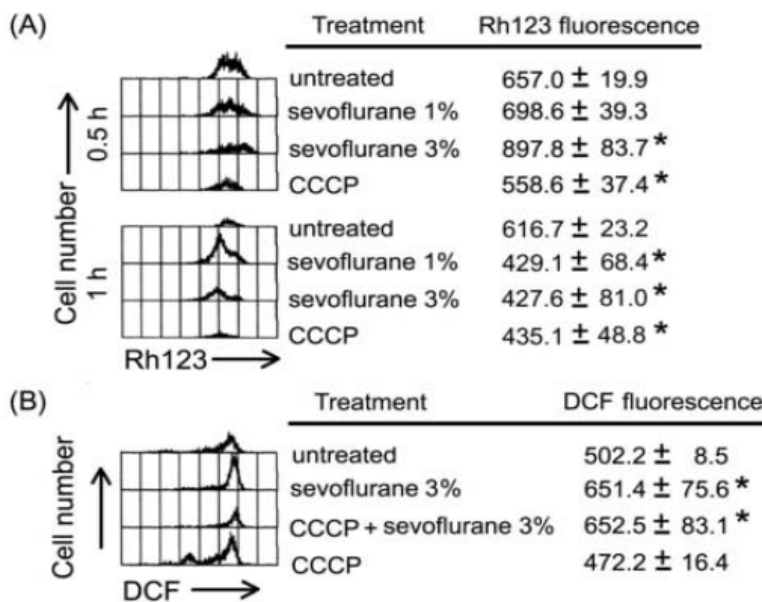 by centrifugation, and suspended in the same buffer. The DCF fluorescence reflecting the level of intracellular H_2O_2 and/or peroxide in PMN was measured by flow cytometry. The data represents the DCF fluorescence intensity within the PMN. The values shown are mean±standard errors (n=3 of individual experiments). Significant differences from the untreated group and sevoflurane alone group are $p < 0.05$ (*) and $p < 0.05$ (#), respectively.

Fig. 7. (A) Effect of sevoflurane on $\Delta\Psi_m$ in PMN. PMN (10^6 cells/ml) were preincubated in 5% CO_2 incubator for 15 minutes with 5 μM rhodamine 123. PMN were then stimulated with 1% and 3% sevoflurane for 0.5 and 1 h in 37^0C CO_2 incubator. For $\Delta\Psi_m$ disrupt group, PMN were treated with 1 μM CCCP for 1h. After drugs treatment, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The rhodamine 123 fluorescence reflecting the level of $\Delta\Psi_m$ in PMN was measured by flow cytometry. The data represents the rhodamine 123 fluorescence intensity within the PMN. The values shown are mean± standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*). (B) Effect of CCCP on sevoflurane-induced H_2O_2 and/or peroxide in PMN. PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator with incubation buffer. PMN were first pretreated with 1 μM CCCP for 45 minutes, and then preincubated with 20 μM DCFH-DA for another 15 minutes, and finally treated with 3% sevoflurane for 1 h. After drugs treatment, PMN were washed once with incubation buffer, collected by centrifugation, and suspended in the same buffer. The DCF fluorescence reflecting the level of intracellular H_2O_2 and/or peroxide in PMN was measured by flow cytometry. The data represents the DCF fluorescence intensity within the PMN. The values shown are mean±standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*).
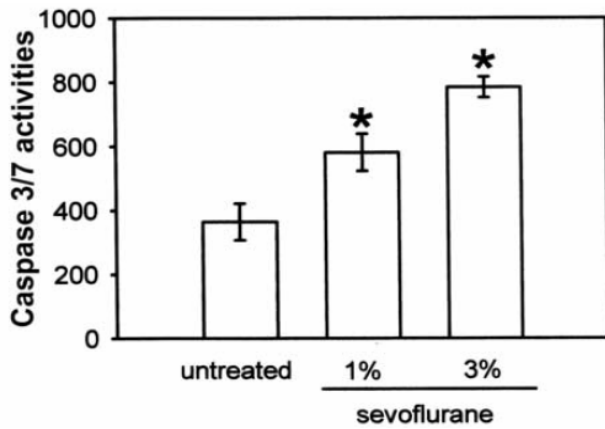
Fig. 8. Effect of sevoflurane on intracellular caspase 3/7 activities in PMN. PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator for 1 h with 1% and 3% sevoflurane. After drugs treatment, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in a DMEM medium, then homogeneous substrate reagent, containing Z-DEVD-R110 were added to the cells, maintaining a 1:1 ratio of reagent to cell solution. After 2 h of incubation at 37^oC, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in incubation buffer. Substrate cleavage to release free R110 fluorescence intensity was measured by flow cytometry. The data represents the intracellular caspase 3/7 activities within the PMN. The values shown are mean±standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*).
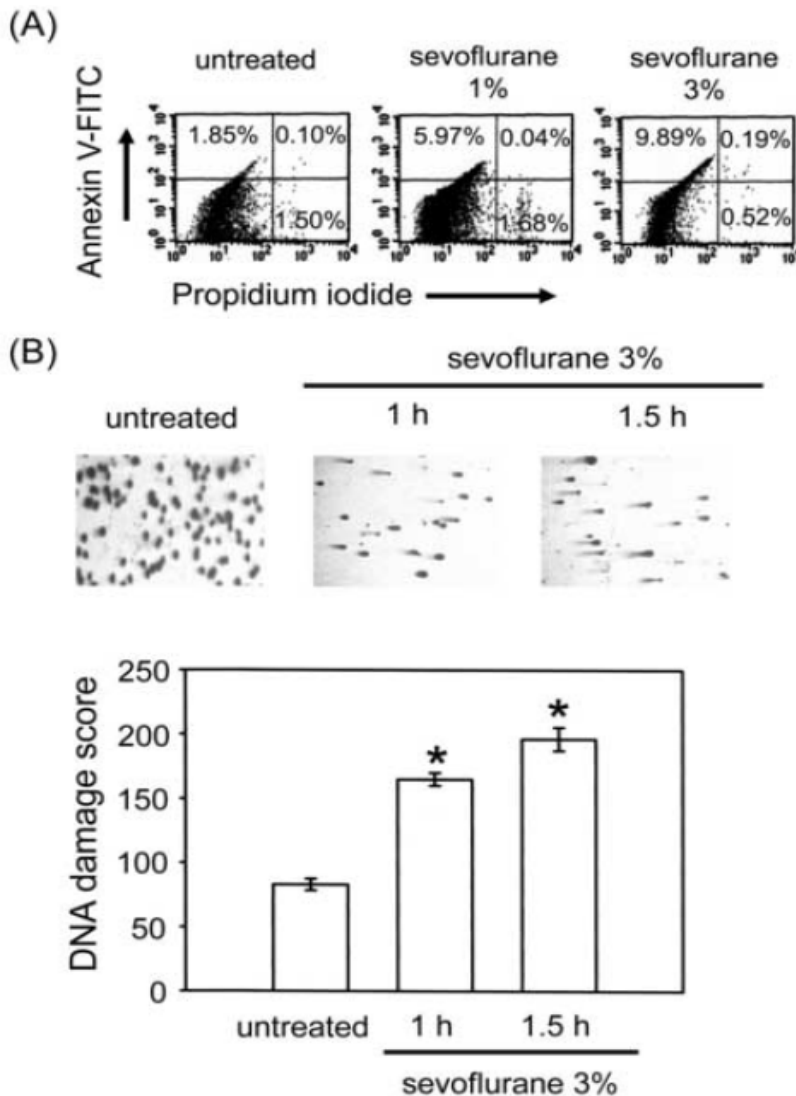

Fig. 9. Effect of sevoflurane on apoptosis and necrosis in PMN. (A) PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator for 2 h with 1% and 3% sevoflurane. After drugs treatment, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in an annexin V-FITC-propidium iodide reactive solution. After 15 minutes of incubation at room temperature, the cells were washed once with incubation buffer, collected by centrifugation, and suspended in incubation buffer. The percentages of apoptotic and necrotic PMN were measured by flow cytometry. Values in the upper left region, lower right region, and upper right region, represent the apoptotic PMN, necrotic PMN, and apoptosis and necrosis coexisted PMN, respectively. (B) PMN (10^6 cells/ml) were incubated in 5% CO_2 incubator for 1 and 1.5 h with 3% sevoflurane. After drugs treatment, the cells were washed once with incubation buffer, collected by centrifugation, and comet assay was preformed as described under "Materials and Methods". PMN with damaged DNA in various fields were counted and the average was taken. Data are shown as mean±standard errors (n=3 of individual experiments). Significant differences from the untreated group are $p < 0.05$ (*).

計畫成果自評部份：

研究內容與原計畫相符程度……………………………………………………………………完全相符

達成預期目標情況……………………………………………………………………完全達成預期目標

研究成果之學術或應用價值…………………………具學術發表價值、可發表於國際期刊

是否適合在學術期刊發表或申請專利…………………………適合發表於國際期刊

主要發現或其他有關價值等：本計畫發現七氟烷會導致人類多形核白血球產生過氧化氫、超氧自由基及一氧化氮等自由基、消耗細胞抗氧化物質--穀胱甘肽、造成粒線體膜電位下降、並誘導細胞凋亡及劊蛋白酶活化，值得作為臨床使用之參考。